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Urease-Independent Chemotactic Responses of *Helicobacter pylori* to Urea, Urease Inhibitors, and Sodium Bicarbonate

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Helicobacter pylori **CPY3401 and an isogenic urease-negative mutant, HPT73, showed chemotactic responses to urea, flurofamide (a potent urease inhibitor), and sodium bicarbonate. Since urea and sodium bicarbonate are secreted through the gastric epithelial surface and hydrolysis of urea by urease on the bacterial surface is essential for colonization, the chemotactic response of *H. pylori* may be crucial for its colonization and persistence in the stomach.**

Helicobacter pylori is one of a few species of bacteria which colonize the gastric mucus layer of humans. Since the bacterium induces a chronic inflammatory response in the stomach that can result in peptic ulceration and gastric neoplasms (3), it is important to understand the mechanisms by which *H. pylori* colonizes and persists in the gastric mucus layer. *H. pylori* is highly motile and is characterized by a spiral body with a bundle of unipolar sheathed flagella. High motility even in a viscous environment (7) is a virulence determinant of *H. pylori*. The cells' sheathed flagella contain two flagellin molecules, FlaA, the major species, and FlaB, which is expressed in minor amounts (9). Both flagellin species are necessary for full motility on soft agar plates and for full colonization of gnotobiotic piglets (5). The bacterium is also unique in having a large amount of urease in the cytoplasm and on the cell surface (8, 15). A *ureB*-disrupted mutant was unable to colonize the stomachs of nude mice, which was ascribed to the failure of ammonia production for neutralization of the acidic microenvironment (17). Urease-producing *H. pylori* cells survived in vitro at pH 2 in the presence of urea, but not in its absence, suggesting that exogenous urea is required for acid resistance (18). These findings indicated that urea is important for *H. pylori* infection. Therefore, we hypothesized that *H. pylori* has the ability to access urea by means of flagellar movement. In the present study, we report the first example of bacterial chemotaxis with urea and bicarbonate as attractants.

Bacterial strains and chemotaxis assay. *H. pylori* CPY3401 and one of its *ureB*-disrupted mutants, HPT73 (17), were used. Bacteria were grown on brucella agar supplemented with 5% horse serum under microaerobic conditions at 37°C for 2 days and were carefully suspended in the chemotaxis buffer (10 mM potassium phosphate buffer [pH 7.0]) to a concentration of 3×10^8 cells per ml (optical density at 560 nm of 0.4). Chemotaxis (swimming) was assayed by a modification of the procedure described by Adler (1). One-microliter micropipettes (Microcaps; Drummond Scientific Co., Broomall, Pa.) were sealed at one end and filled with various compounds in the chemotaxis buffer to make chemoattractant tubes. A small chamber formed by laying V-shaped sealed micropipettes between a microscope slide and a coverslip was filled with 200 μl of bacterial suspension. Three chemoattractant tubes were then placed into the chamber to be in contact with the bacterial suspension. The setting up of the chamber and subsequent incubations were routinely carried out at 28°C. The chemotaxis assay at 37°C was technically difficult because of evaporation during the procedure. After incubation, bacteria in the tube were spread over a known area (0.125 cm^2) on a microscope slide, Gram stained, and counted under a microscope. Data were expressed as means (error bars indicate standard deviations) for three determinations.

The strains retained their motility and colony-forming ability in the chemotactic buffer after incubation for 120 min at 28°C. Since urea is a rapidly diffusing molecule, we measured the urea concentration in the tube before and after a 60-min incubation without bacteria by the method of Creno et al. (4). The urea concentration in the tube containing 10 mM urea decreased to 6.3 mM after the incubation, suggesting that the urea diffused from three 1-μl tubes (3.7 mM urea in 3 μl) in the 200-μl chamber might not have a significant effect on the chemotaxis assay.

Chemotactic response to urea and its analogs. *H. pylori* CPY3401 entered the tubes containing 1 and 10 mM urea, and a higher concentration of urea (100 mM) repressed the migration (Fig. 1A). The average bacterial count per tube in the absence of urea was 14, whereas those in the presence of 1 mM urea at 60 min and 10 mM urea at 30 min were 350 and 640, respectively. Since urea hydrolysis by bacterial urease might have some effects on the chemotactic response, we carried out experiments to estimate (i) the change in the pH of the solution in the tube containing 10 mM urea during the incubation, (ii) the amounts of the reaction products NH_3 and CO_2 necessary to cause the pH change, and (iii) the chemotactic response of the bacteria to NH_4Cl and $NaHCO_3$. We obtained the following results. (i) The pH was approximately 7.2 after the 60-min incubation. (ii) The complete hydrolysis of 1 mM urea in the chemotaxis buffer by Jack bean urease changed the pH of the chemotaxis buffer from 7.02 to 7.23. (iii) The average bacterial counts per tube, which contained 1 mM NH_4Cl or 1 mM $NaHCO_3$, were 53 and 300, respectively (see below). The last findings indicated that we could not discount the effect of urea hydrolysis on the chemotactic response toward urea. Therefore, we carried out similar experiments with the isogenic urease-negative strain HPT73. The latter bacteria were also attracted by urea but less efficiently than the urease-positive bacteria (Fig. 1B). The average bacterial counts in the absence and in the presence of 10 mM urea at 60 min were 14 and 300, respectively. We carried out an additional experiment with HPT73 to see the effect of urea which was present both in the

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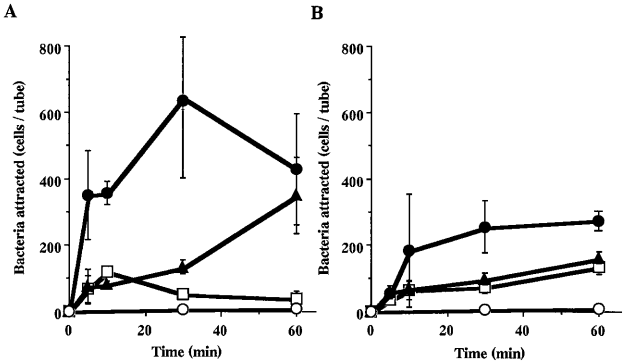

FIG. 1. Chemotactic response of *H. pylori* CPY3401 (A) and HPT73 (B) to urea. Data had higher standard deviations than those of other chemotactic responses as shown in Fig. 2 and 3. Urea concentrations: ○, none; ▲, 1 mM; ●, 10 mM; □, 100 mM.

TABLE 1. Chemotactic responses of *H. pylori* to urea analogs

Urea analog	No. of bacteria/tube (mean ± SD) at 60 min	
	CPY3401	HPT73
None	13.7 ± 1.52	49.7 ± 61.25
Hydroxyurea		
1 mM	115 ± 38.11	165 ± 55.23
10 mM	159 ± 27.02	116 ± 37.47
Acetohydroxamic acid		
1 mM	220 ± 27.43	213 ± 20.11
10 mM	182 ± 74.19	138 ± 43.82
Ammonium chloride		
1 mM	53 ± 29.05	241 ± 87.30
10 mM	77 ± 7.94	107 ± 77.57

tubes and on the slide. The average bacterial count was 28 at 30 min in the presence of 10 mM urea both in the tubes and on the slide. Based on these results, we concluded that *H. pylori* has chemotactic activity toward urea.

Bacteria of strains CPY3401 and HPT73 were also attracted to a urease inhibitor, *N*-(diaminophosphinyl)-4-fluorobenzamide (flurofamide; Tocris Cookson Ltd., Bristol, United Kingdom) (Fig. 2). The concentration of flurofamide to give maximum migration was 1 μM for both strains, and a higher concentration (10 μM) repressed the activity. The chemotactic response of CPY3401 to 1 μM flurofamide at 37°C was similar to the response at 28°C within 30 min, while the bacterial counts leveled off subsequently (data not shown). When 1 μM flurofamide was present both in the suspension buffer and in the tube, the bacterial migration was not observed. Urea analogs such as acetohydroxamic acid, hydroxyurea, and, possibly, ammonium chloride also attracted the bacteria (Table 1). The concentrations of urease inhibitors for chemotactic response of both urease-positive and urease-negative strains were comparable to the 50% inhibitory doses of *H. pylori* urease (12). The potent inhibitory effect of flurofamide on urease was ascribed to the resemblance of the phosphorotriamide portion of the structure to the tetrahedral intermediate of urea in the urea-urease interaction (11). This structural mimicry might reflect the behavior of urea-responsive bacteria toward flurofamide. Previously we showed that neither UreA nor UreB subunits are present in the cells of a *ureB*-disrupted strain (13). Therefore, the findings presented above suggest that *H. pylori* has a urea-responsive molecule for locomotion which is independent of the urease subunits.

In contrast to urea and its analogs, L-glutamine or glycine did not attract the bacteria; the average counts of CPY3401 with 1 mM glutamine and 1 mM glycine were 58 and 36, respectively. Some amino acids such as glutamate and methionine at a concentration of 1 mM attracted the bacteria (data not shown).

Chemotactic response to sodium bicarbonate. Bacteria of *H. pylori* CPY3401 migrated into tubes containing sodium and potassium bicarbonate (Fig. 3). The maximal migration was observed in the presence of 10 mM (each) sodium and potassium bicarbonate. In addition, the bacteria were attracted by sodium chloride (Fig. 4A), whereas the attraction by potassium chloride was not prominent (Fig. 4B). The chemotactic response to sodium chloride was not inhibited by 1, 2.5, and 5 mM amiloride, a Na^+ pump inhibitor, suggesting that the energy source for the flagellar movement may be the proton motive force, but not the sodium motive force which drives the polar flagellar motor of *Vibrio parahaemolyticus* (2). Similar results were obtained with urease-negative HPT73 (data not shown).

The chemotactic response of bacteria starts by sensing a concentration gradient of attractants by a sensor molecule on the cell membrane, and the signal is transduced to the locomotion machinery, which makes the bacteria move toward a

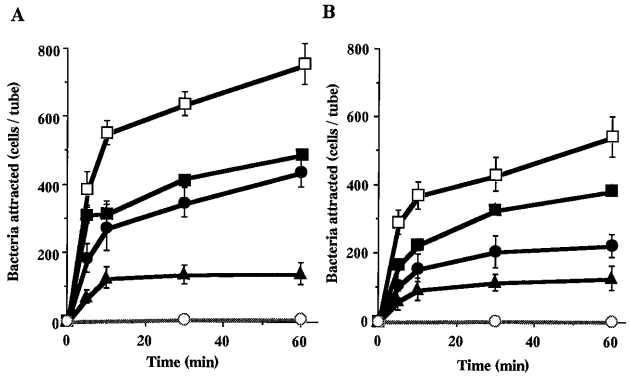

FIG. 2. Chemotactic response of *H. pylori* CPY3401 (A) and HPT73 (B) to flurofamide. Flurofamide concentrations: ○, none; ▲, 0.1 μM; ●, 0.25 μM; □, 1 μM; ■, 10 μM.

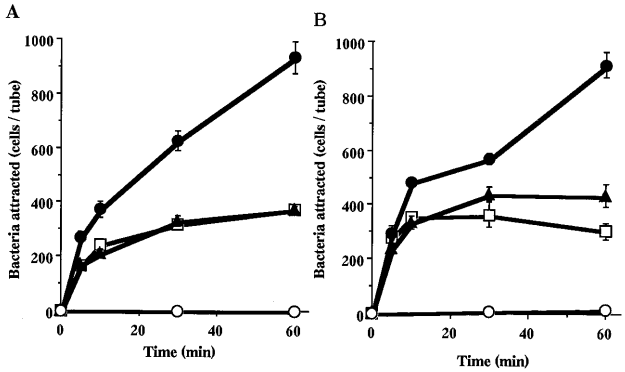

FIG. 3. Chemotactic response of *H. pylori* CPY3401 to sodium bicarbonate (A) and potassium bicarbonate (B). Concentrations: ○, none; ▲, 1 mM; ●, 10 mM; □, 100 mM.

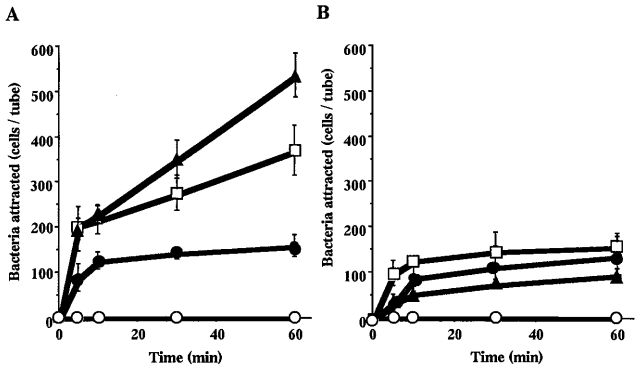

FIG. 4. Chemotactic response of *H. pylori* CPY3401 to sodium chloride (A) and potassium chloride (B). Concentrations: ○, none; ▲, 1 mM; ●, 10 mM; □, 100 mM.

higher concentration of attractants (6). Urea is synthesized exclusively in the liver and is circulated and excreted in the gastric juice. Finely developed capillary networks beneath the basement membrane of epithelial cells supply urea, and a concentration gradient of urea must be formed in the mucus layer. The urea concentrations in the sera and gastric juices of healthy uninfected subjects are, on average, 5 and 2 mM, respectively (14). Our results indicated that these concentrations are in the effective range to attract *H. pylori*. Furthermore, the chemotactic response to urea was observed in a viscous environment of 3% polyvinylpyrrolidone (19). Therefore, *H. pylori* in the mucus layer may sense urea and move toward the epithelial cell surface, which must be important for persistent infection of this organism. In accordance with this, Kirshner and Blaser (10) assumed that *H. pylori* resides mostly in the gastric mucus layer and that only a small portion (2%) attaches to the epithelial cells, so, therefore, the former population is present to replenish the latter. *H. pylori* cells from young cultures have urease in the cytoplasm, and the surface localization of urease is ascribed to autolysis of a portion of the bacteria in the population (15). *H. pylori* cells with surface urease may hydrolyze urea rapidly before sensing. We assume that *H. pylori* cells without surface urease may be swimmer cells which sense urea, and the cells with surface urease may be produced after colonization. The chemotactic response of *H. pylori* to urea ensures that urease will produce ammonia and carbon dioxide. Ammonia neutralizes gastric acid to form ammonium chloride, which in turn serves as a nitrogen source. Thus, the chemotactic response to urea should be crucial not only for acid resistance, but also for colonization in the hostile environment.

Bicarbonate is secreted into the gastric mucosa by a chloride-bicarbonate exchanger localizing in parietal cells whereas Na^+ is secreted by a Na^+-H^+ exchanger localizing in the mucous neck, chief, and surface mucous cells (16). The chemotactic response to sodium bicarbonate may also contribute to the persistence of *H. pylori*. Since bicarbonate anion is one of the reaction products of urease, this response might be important in the absence of urea. Thus, the chemotactic response to bicarbonate may assist the chemotaxis with urea.

The chemotactic response to urea appears to be specific for *H. pylori*. In preliminary experiments, we observed no such activity in *Helicobacter fennelliae*, a highly motile, urease-negative species of bacteria which was isolated from intestine. Similarly, some enteric bacteria, such as *Proteus mirabilis* and *Escherichia coli*, did not show a significant response to urea (data not shown). Further studies are necessary to characterize the unique chemotactic response in *H. pylori* in detail in order to understand the molecular mechanism and pathobiological significance of bacterial ureataxis.

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